Chapter 31

Measurement of Mitochondrial Toxicity Parameters in Embryonic Hippocampus

Ahmad Salimi and Jalal Pourahmad

Abstract

Recent discoveries have focused on mitochondria functions in the neuroscience research for approaches to study mitochondria dysfunction in neurodegenerative diseases. Mitochondrion is one of the organelles that is possibly worst affected in cognitive impairments. These are known as "powerhouse" of the cell as they are the main source of generation of ATP through aerobic respiration. They have role in oxidative phosphorylation and metabolism, they play central role in cell differentiation, apoptosis, oxygen sensing and detoxification of reactive oxygen species, innate immunity, mitochondrial matrix calcium, and maintenance of cell quality and regulation of cytoplasmic. There is a relationship between mitochondrial dysfunction and cognitive disorder that may be related to certain neurotoxins or mutations in mitochondrial DNA as well as the nuclear. Evaluating compounds for mitochondrial toxicity is an important capability for evaluation of cognitive effects by drugs. Studying mitochondria isolated from individual mouse brain regions is a challenge because of small amount of the available brain tissue. There are conventional techniques for isolation and purification of mitochondria from ventral midbrain, hippocampus, or striatum. The utilization of alcohol within pregnancy impairs the development of the unborn offspring and can lead to a plethora of anatomical, behavioral, and cognitive abnormalities.

In here, a method for isolation of brain mitochondria from mouse is described. The method utilizes a refrigerated tabletop microtube centrifuge, and produces research grade quality mitochondria in amounts sufficient for performing multiple enzymatic and functional assays, thereby eliminating the necessity for pooling mouse brain tissue. A method for measuring ROS measurement, mitochondrial membrane potential, mitochondrial swelling, cytochrome *c* release, and mtDNA alterations after exposure to drugs is also included.

Key words Mitochondria, Embryonic hippocampus, Oxidative stress, Cognitive effects, Drugs

1 Introduction

Mitochondria are pleomorphic and dynamic organelles. The main role of mitochondria is the production of energy as adenosine triphosphate, heat production by decoupling of the oxidative phosphorylation, translation and transcription of mitochondrial genes, and induction of cell death by apoptosis [1]. Mitochondria play a main role in apoptosis, and mitochondrial dysfunction emerges to

Luís Félix (ed.), *Teratogenicity Testing: Methods and Protocols*, Methods in Molecular Biology, vol. 1797,
https://doi.org/10.1007/978-1-4939-7883-0_31,

have a certain impact on the pathogenesis of neurodegenerative diseases. Mitochondrial dysfunction is a hallmark of many disorder that cause brain impairments and impinge on cognitive function, such as neurodegenerative diseases, aging and genetic mitochondrial disorders [2, 3]. Research has been directed at elucidating the involvement of mitochondria and defects in mitochondrial oxidative phosphorylation in late-onset neurodegenerative disorders. Numerous studies have now uncovered divergent roles for mitochondria that go well beyond ATP production. The major class of drugs to treat affective spectrum disorders has been evidenced to affect mitochondrial function; for example new-generation antidepressive therapeutics have preferential action on mitochondrial metabolism [4]. The toxicity of many drugs is manifested through the production of reactive oxygen species (ROS) that may lead to mitochondrial damage. Therefore, the evaluation of mitochondrial health in postnatal mouse could be effective in determination of induced damages by new candidate drugs and other chemicals.

In this chapter a method for isolation of brain mitochondria that allows the isolation of a workable amount of mitochondria from 7 to 12 mg brain samples is described. In addition, the most useful and frequently assessed mitochondria functional characteristics such as ROS formation, mitochondrial membrane potential, mitochondrial swelling, cytochrome c release, and mtDNA damage are described.

2 Materials

2.1 Brain Mitochondria Isolation

1. A surgical set for removing of brain.
2. Kontes glass homogenizer, small clearance pestle.
3. Bucket filled with ice.
4. A refrigerated microtube centrifuge.
5. Microcentrifuge tubes (*see* **Note 1**).
6. Glass or plastic dissection support surface such as Petri dish and a piece of Whatman paper.
7. Plate reader capable of measuring fluorescence and absorbance multiwavelengths.
8. Pipettor and tips.
9. Mannitol–sucrose–EGTA (MSEGTA) buffer: 225 mM mannitol, 75 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, dissolved in water (*see* **Note 2**). Store between 0 and 4 °C (*see* **Note 3**).
10. MSEGTA–BSA: MSEGTA buffer supplemented with 0.2 mg/mL bovine serum albumin (BSA) essentially fatty acid-free. Store between 0 and 4 °C.

11. 100% Percoll™–MSEGTA buffer: 225 mM mannitol, 75 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, dissolved in 100% Percoll™. Store between 0 and 4 °C.
12. Experimental buffer: 8 mM KCl, 110 mM potassium gluconate, 10 mM NaCl, 10 mM HEPES (acid), 10 mM KH_2PO_4 0.005 mM EGTA, 10 mM mannitol, 1.5 mM $MgCl_2$, 0.5 mg/mL BSA essentially fatty acid-free, pH 7.25 (adjusted with KOH). Store at 4 °C.
13. 0.5 M EDTA.
14. 1 M $MgCl_2$.

2.2 Measurement of Mitochondrial Concentration

1. Bovine serum abumin (BSA) (*see* **Note 4**).
2. Coomassie Brilliant Blue G-250.
3. Methanol.
4. Phosphoric acid (H_3PO_4).
5. Bradford reagent.
6. Spectrophotometer.
7. Whatman paper.

2.3 ROS Formation

1. Isolated mitochondria (*see* **Note 5**).
2. Incubation buffer: 125 mM KCl, 4 mM KH_2PO_4, 14 mM NaCl, 20 mM HEPES–NaOH, pH 7.2, 1 mM $MgCl_2$, 0.2% of fatty acid-free bovine serum albumin, and 0.020 mM EGTA.
3. 10 μM 2′,7′-dichlorodihydrofluorescein (DCF): Prepare a stock solution of DCF at 10 mM in dimethylsulfoxide (DMSO). Further dilute it to 10 μM in DMSO.
4. H_2O_2 solution in water, 30–32 wt.%.
5. Respiratory substrates (5 mM sodium succinate) and inhibitors (2 μM Rotenone).
6. Small (5–10 mL) glass tubes (*see* **Notes 7** and **8**).
7. Glass (preferable) or plastic disposable cuvettes with all walls transparent and flat bottom and matching stirring bars.
8. A quartz cuvette.
9. Fluorimeter equipped with a stirred thermostated cuvette holder.
10. Common labware such as pipettors and tips.

2.4 Mitochondrial Membrane Potential (MMP) Collapse Measurement

1. Isolated mitochondria (*see* **Note 5**).
2. MMP assay buffer: 220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 μM EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 μM Rotenone.
3. 10 μM Rhodamine 123: Prepare a stock solution at 10 mM in dimethylsulfoxide (DMSO). Further dilute it to 10 μM in DMSO.

4. Respiratory substrates (5 mM sodium succinate) and inhibitors (2 μM Rotenone).
5. Small (5–10 mL) glass tubes (*see* **Notes 7** and **8**).
6. Glass (preferable) or plastic disposable cuvettes with all walls transparent and flat bottom and matching stirring bars.
7. A quartz cuvette.
8. Fluorimeter equipped with a stirred thermostated cuvette holder.
9. Common labware such as pipettors and tips.

3 Methods

1. Anesthetize postnatal mice with an intraperitoneal injection (800 μL) of pentobarbital and perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 at 4 °C.
2. Remove the brain from the skull and place excised brain on the ice-chilled glass or plastic surface (a petri dish) covered with wet Whatman paper; wash out the blood out of the brain surface (use mitochondria isolation buffer to wash and to wet the paper).
3. Using sterile scissors or scalpel, open cranium of pup from back of the neck to the nose. Carefully remove the entire brain with forceps. Place the brain on sterile gauze. Using a sterile scalpel, remove the cerebellum and incise down the midline of the brain to separate it into two hemispheres (*see* Fig. 1a of Chapter 30).
4. Grasp a small section of meninges surrounding the hippocampus with sterile forceps and pull it gently away. In either case, the hippocampus will be more clearly visible after the meninges have been removed. The hippocampus is a curved structure that starts in the distal part of the hemisphere and bends ventrally (*see* Fig. 1b of Chapter 30).
5. Immediately place it in 1 mL of ice-cold MSEGTA-BSA buffer.
6. Homogenize hippocampus tissue obtained in step and add 1 mL MSEGTA-BSA with 2 mL homogenizer, tight pestle, with 30–35 strokes, transfer into two 1.7 mL microcentrifuge tubes.
7. Add MSEGTA-BSA to the tubes up to the groove at the top (~1.7 mL, total volume), mix by pipetting or inversion.
8. Centrifuge at ~500 × *g* × 5 min. Transfer the supernatant into clean 1.7 mL microcentrifuge tubes and centrifuge at 14,000 × *g* × 10 min.

9. While centrifuging at **step 7**, fill 1.7 mL microcentrifuge tube with 1 mL of 24% Percoll MSEGTA, and prepare 12% Percoll™-MSEGTA, 0.2 mL (take 0.1 mL of 24% Percoll™-MSEGTA and dilute with 0.1 mL MSEGTA) and keep both 24% and 12% Percoll™-MSEGTA-filled tubes in ice.
10. Aspirate off the supernatant and resuspend both pellets (combine them) in 0.2 mL of 12% Percoll™-MSEGTA.
11. Carefully layer the suspension over the 24% Percoll™-MSEGTA solution, by holding the tube at about 30°, placing the tip of the pipettor into the tube's groove and slowly releasing the suspension. Close the tube, turn it upright and place in ice before proceeding to the next step. Fill another 1.7 mL microcentrifuge tube with 1.2 mL of MSEGTA to use as a counterbalance in the centrifuge.
12. Centrifuge at 18,000 × *g* × 15 min. After the centrifugation is completed, check the appearance of the sample. There should be almost transparent band approximately in the middle of the tube, with cloudy top and bottom portions of the sample.
13. Aspirate off 0.7 mL of the top portion of the sample.
14. Add 1.2 mL MSEGTA, mix by inversion, and centrifuge at 18,000 × *g* × 5 min.
15. Aspirate 1.5 mL of the supernatant, resuspend the pellet in the remaining 0.2 mL of the isolation buffer, add 1.5 mL MSEGTA, mix by inversion and centrifuge at 14,000 × *g* × 5 min.
16. If the pellet is fuzzy, repeat previous **step 14**.
17. If the pellet appears solid with well-defined shape, aspirate off the supernatant completely and resuspend the pellet in 0.1 mL MSEGTA. This is purified mitochondrial fraction (*see* **Note 3**).

3.1 Mitochondrial Concentration

3.1.1 Bradford Reagent Preparation

1. Dissolve 50 mg of Coomassie Brilliant Blue G-250 in 50 mL of methanol and add 100 mL 85% (w/v) phosphoric acid (H_3PO_4).
2. Add the acid solution mixture slowly into 850 mL of H_2O and let the dye dissolve completely.
3. Filter using Whatman #1 paper to remove the precipitates just before use. Store in a dark bottle at 4 °C.

3.1.2 Measurement of Mitochondrial Concentration

1. Prepare five dilutions of a protein (usually BSA) standard with a range of 5–100 μg protein.
2. Dilute unknown protein samples to obtain 5–100 μg protein/30 μL.
3. Add 30 μL of each standard solution or unknown protein sample to an appropriately labeled test tube.

4. Set two blank tubes. For the standard curve, add 30 μL H_2O instead of the standard solution. For the unknown protein samples, add 30 μL protein preparation buffer instead. Protein solutions are normally assayed in duplicate or triplicate.
5. Add 1.5 mL of Bradford reagent to each tube and mix well.
6. Incubate at room temperature (RT) for at least 5 min. Absorbance will increase over time; samples should incubate at RT for no more than 1 h.
7. Measure absorbance at 595 nm.
8. Prepare five standard solutions (1 mL each) containing 0, 10, 20, 30, 40 and 50 μg/mL BSA.
9. Pipet 800 μL of each standard and sample solution (containing for <50 μg/mL protein) into a clean, dry test tube. Protein solutions are normally assayed in duplicate or triplicate.
10. Add 200 μL of dye reagent concentrate to each tube and vortex.
11. Follow the procedure described above for the standard assay procedure.

3.2 ROS Measurement

3.2.1 H_2O_2 Calibration Solution

1. Fill two tubes with 5 mL of deionized water.
2. To the first tube, add 5 μL of 30% (v/v) H_2O_2 solution (1:1000 dilution), mix well.
3. Determine the concentration of H_2O_2 spectrophotometrically by placing 2 mL of this solution in a quartz cuvette and reading its absorbance at 240 nm; calculate the concentration of H_2O_2 employing the extinction coefficient $\varepsilon 240 = 43.6/M/cm$.
4. Dilute H_2O_2 solution to ~0.1 mM by taking 50 μL of H_2O_2 solution from this tube and adding it to the second tube (1:100 dilution), mix well.
5. Keep the second tube in ice and use within 1 h to calibrate the H_2O_2 assay.

3.2.2 Mitochondrial H_2O_2 Emission

1. Set the fluorimeter at 488 nm excitation and 527 nm emission wavelengths.
2. Turn on the cuvette holder's thermostat set at the desired temperature (25–37 °C).
3. Fill a cuvette with the incubation buffer, add magnetic stirring bar, turn on the stirrer, and wait until the cuvette reaches the desired temperature (25–37 °C).
4. Add 10 μM DCF and mitochondria (0.03–0.1 mg/mL) to the cuvette.

5. Record the fluorescence for ~150 s.
6. The data could be presented by % of control and fluorescence intensity (*see* **Note 6**).

3.3 MMP Collapse Measurement

1. Set the fluorimeter at 490 nm excitation and 535 nm emission wavelengths.
2. Turn on the cuvette holder's thermostat set at the desired temperature (25–37 °C).
3. Fill a cuvette with the incubation buffer, add magnetic stirring bar, turn on the stirrer, and wait until the cuvette reaches the desired temperature (25–37 °C).
4. Add 10 μM Rhodamine 123 and mitochondria (0.03–0.1 mg/mL) to the cuvette.
5. Record the fluorescence for ~150 s.
6. The data could be presented by % of control and fluorescence intensity (*see* **Note 7**).

4 Notes

1. Eppendorf tubes can be substituted with any other brand of small polypropylene tubes of similar volume, with a conical bottom. For all aliquoted reagents, use only noncolored, clear plastic tubes.
2. All water solutions and reagents are prepared using distilled water.
3. The frozen stocks of the mitochondrial substrates are stable for at least 1 year. It is difficult to prepare K^+ malate stocks at a concentration higher than 0.5 M.
4. The experimental buffer contains BSA, and therefore it tends to foam. While pipetting materials in the well, care must be taken to avoid extra foaming, that may affect the fluorescence signal.
5. Quality of and purity of mitochondria is very important.
6. Contamination of mitochondrial preparation with cytosolic structures such as peroxisomes, synaptic terminals, etc. and with fragments of broken mitochondria can significantly interfere with ROS and MMP collapse measurements.
7. The other mitochondrial toxicity parameters such as mitochondrial swelling, cytochrome c and ATP/ADP ratio also could be measured in isolated mitochondria obtained hippocampus using appropriate protocols and ready kits.

References

1. Reeve AK, Krishnan KJ, Turnbull D (2008) Mitochondrial DNA mutations in disease, aging, and neurodegeneration. Ann N Y Acad Sci 1147(1):21–29. https://doi.org/10.1196/annals.1427.016
2. Petrozzi L, Ricci G, Giglioli NJ et al (2007) Mitochondria and neurodegeneration. Biosci Rep 27(1-3):87–104
3. Winklhofer KF, Haass C (2010) Mitochondrial dysfunction in Parkinson's disease. Biochim Biophys Acta 1802(1):29–44. https://doi.org/10.1016/j.bbadis.2009.08.013
4. Krivakova P, Cervinkova Z, Lotkova H et al (2005) Mitochondria and their role in cell metabolism. Acta medica (Hradec Kralove) Suppl 48(2):57–67